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Short Sequence-Paper

Bacillus amyloliquefaciens possesses a second type I signal peptidase with extensive sequence similarity to other *Bacillus* SPases ☆

Viet Hoang, Jürgen Hofemeister *

Institute of Plant Genetics and Crop Plant Research, Department of Molecular Genetics, Gatersleben, D-06466, Germany

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Abstract

A second *sipS2(BA)* gene was PCR cloned from *Bacillus amyloliquefaciens*. The deduced aa sequence is similar to those of the SPases of *B. subtilis*, *B. amyloliquefaciens*, and *B. licheniformis* and the domain structure of the gene has been preserved. A low level of monocistronic gene transcription could be shown using Northern analysis. The *sipS2(BA)* gene was mapped to a region downstream of an *E. coli fruA* gene homologue and shown to express a 21 kDa protein in *Escherichia coli*.

Keywords: Signal (leader) peptidase type I; *sip* gene; PCR cloning; Nucleotide sequence; Sequence alignment; *fruA* gene; (*Bacillus*)

Signal peptidases (SPases) are membrane-bound proteinases that remove targeting (signal) peptides from secretory (pre-)proteins and play a central role in the translocation of proteins through membranes [1–3]. They constitute a new (serine) proteinase family with unique substrate specificity and limited sequence similarity [4–6]. Although the *sipS* gene was shown to encode a type I SPase in *Bacillus subtilis*, its amino-acid sequence, domain structure, and substrate specificity were distinct from those of *Escherichia coli* Lep(EC), *Salmonella typhimurium* Lep(ST), and *Pseudomonas fluorescens* (LepPF) counterparts [3,4,7]. Moreover, Van Dijl et al. [8] have proposed the existence of two type I SPases in *B. subtilis* from the fact that a *sipS* gene deletion mutant could still process and secrete proteins. Here we report the cloning and characterisation of a second SPase-like gene *sipS2(BA)* from *B. amyloliquefaciens*. We show that the gene is transcribed and that it codes for a putative second SPase (isozyme) in *B. amyloliquefaciens*.

PCR amplification of sipS core DNA by homology. The published aa sequence of a *B. subtilis* SipS(BS) protein [8] was used as the basis for the generation of two degenerate oligonucleotide primers A and B (Table 1). The PCR reactions were carried out using genomic DNA from *B. amyloliquefaciens* strain BE71 (a rifampicin resistant derivative of strain ATCC 23 844). A DNA core region of approx. 300 bp was specifically amplified using primers A and B (Fig. 1, Table 1). These PCR products were cloned into the pUC18 vector. Several independent clones were sequenced. Two different core sequence inserts were found. One sequence matches that of putative *sipS* gene homologue entry in the EMBL database (EMBL GenBank/DDBJ database accession No. Z27458 submitted by J.M. van Dijl et al., University of Groningen) cloned from *B. amyloliquefaciens* DNA. This putative gene will subsequently be referred to as *sipS1(BA)*. Southern analyses [9], with *Eco*RI and *Hin*dIII digested genomic DNA from *B. amyloliquefaciens* support the existence of the two distinct genes, since two different *Eco*RI and *Hin*dIII fragments, of either 9 and 1.5 kb or 4 and 3.5 kb, respectively, were found to hybridise with our probe (results not shown). Here we report the cloning from genomic DNA of *B. amyloliquefaciens* of a candidate 'second' gene that we have named *sipS2(BA)*.

Abbreviations: aa, Amino acid(s); bp, base pair(s); *fruA*, *E. coli* gene encoding enzyme-II of the fructose specific phospho*enol*pyrovate-dependent sugar phosphotransferase (PTS) system; kb, kilobase(s) or 1000 bp; nt, nucleotide(s); kDa, kilodalton(s); Lep(EC), type I signal peptidase of *E. coli*; Lep(ST), type I signal peptidase of *Salmonella typhimurium*; oligo, oligodeoxyribonucleotide; ORF, open reading frame; PCR, polymerase chain reaction; RBS, ribosome binding site; *sipS*, gene encoding bacterial type I signal peptidase; SipS, product of the *sipS* gene(s); SPase, signal peptidase.

☆ The sequence data reported in this paper has been submitted to the EMBL GenBank/ DDBJ Nucleotide Sequence Database under the accession number Z33640.

* Corresponding author. Tel.: +49 39 4825241; fax: +49 39 482280.

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Table 1
Oligonucleotide primers used for PCR

Primer name	The 5′ → 3′ sequence [a]	Description
A	TT(CT)ATNTT(CT)GCNCCNTA(CT)GTNGT	Degenerate primer [b]
B	TT(GA)TCNCCCATNAC(GA)AA(GA)TA	Degenerate primer [b]
C	AATCTCCCGTCAGGTTGACTCCC	Internal sequence [c] nt 1444–1476
D	TTGCTCGCGAGGTACGGTTCTGC	Internal sequence [c] nt 1400–1431
E	GTACCCGACATTACATGACGG	Internal sequence [c] nt 1204–1224
F	GAACCGTACCTCGCGAGCAAT	Internal sequence [c] nt 1403–1423
Uni1	GTTTTCCCAGTCACGAC	Universal sequencing primer for pUC18
Uni2	GTAAAACGACGGCCAGT	Univeral sequencing primer for pUC18

[a] The nt in parentheses show the alternative, N denotes an inosine residue. The primers were made on an Applied Biosystems 390 Oligonucleotide Synthesizer.
[b] Degenerate primers are designed for the conserved or hydrophobic region of SipS1(BS) from *B. subtilis* [8] encoding aa 32–40 (primer A) or aa 141–147 (primer B) of this protein.
[c] Primers are designed for the core part of SipS2(BA) (see Fig. 5) that was deduced from the PCR amplified core DNA fragment using the degenerate primers A and B (Table 1) and genomic *B. amyloliquefaciens* DNA as template. Primer positions correspond to the nt sequence of the *B. amyloliquefaciens sipS2(BA)* gene region as shown in Fig. 2 of this paper.

Cloning of the sipS2(BA) gene homologue from B. amyloliquefaciens. We were unable to clone the *sipS2(BA)* 3.5 kb fragment DNA into *E. coli* by eluting it from agarose gels and ligating it into *Hind* III cut pUC18 vector DNA (a likely reason for this is discussed later). We therefore attempted to clone the proposed *sipS2(BA)* gene by using the RAGE protocol for PCR cloning [10]. The PCR products of the two reactions using the 'forward' or 'reverse' primers CD or EF, respectively, in combination with the universal primers Uni1 or Uni2 (Table 1, Fig. 1), were either 1.5 or 0.5 kb in size. These fragments were subsequently cloned and sequenced. The two PCR fragments overlapped with the core DNA fragment A-B for about 250 and 60 bp, respectively (Fig. 1). This allowed us to reconstruct a 1914 bp DNA fragment,. The sequence of which is shown in Fig. 2. In order to confirm the existence of an identical segment in the chromosome, suitable primers were made and the expected DNA fragment was indeed amplified from genomic DNA of *B. amyloliquefaciens* (results not shown). The complete ORF2 started with codon TTG at nt 1049 and terminated with a stop codon TAA at nt 1628 (Fig. 2). As expected, the deduced 193 aa-protein exhibited striking homology to the SipS protein of *B. subtilis* as well as to other *Bacillus* type I SPases (Fig. 3, Table 2). Therefore, we conclude that we have cloned of a second *sipS* gene homologue from *B. amy-*

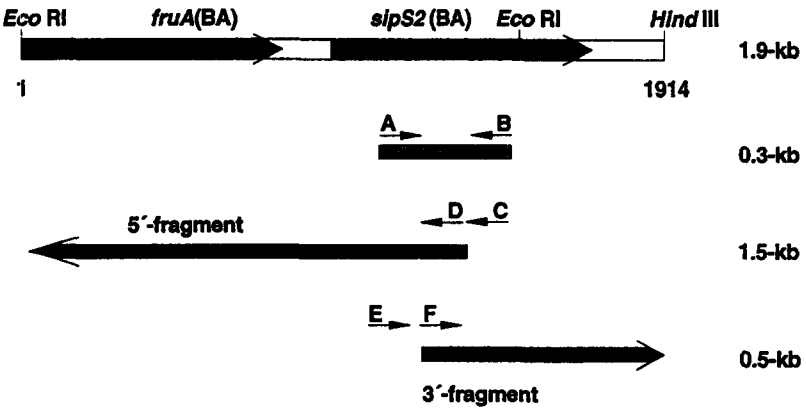

Fig. 1. Cloning strategy according the RAGE protocol [10] is illustrated. Position of the PCR primers A and B used to amplify either the *sipS*-core DNA and the relative orientation of the 'forward' and 'reverse' primers CD and EF that were used to amplify the 5′- and 3′-fragments for alternative PCR are indicated by arrows (see Table 1). The *sipS*-core DNA fragment (A-B) as well as the products of the alternative PCR are shown by shaded bars. The PCR fragments were cloned using the pMOS*Blue* T-vector kit (Amersham Life Science) and sequenced as described in Fig. 2. A tentative restriction map of the reconstructed chromosomal segment and the extension of corresponding ORF regions (shown as dark boxes) are proposed to encode the *sipS2(BA)* gene downstream of or fruA gene homologue in *B. amyloliquefaciens* (see Fig. 2).

loliquefaciens. A comparison of the putative gene revealed striking similarity to the known *B. subtilis sipS(BS)* gene for a putative RBS (GGAGG), their spacing by 10 bp down to the translation start codon that was in every case TTG.

It was interesting to note that a second incomplete ORF consisting of 304 codons exists 74 nt upstream from the *sipS2(BA)* ORF (Fig. 2). The deduced protein sequence exhibited 46% identity to enzyme-II of the *E. coli* fructose specific phospho*enol*pyruvate-dependent sugar phosphotransferase (PTS) system encoded by the *fruA* gene [11]. Althougth the *fruA* gene of *B. subtilis* has not yet been isolated, the *fruAfruB* genes have been mapped to a position close to the *ptsI* locus encoding the enzyme-I of

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GAATTCATTCAGCTGATCCGAAAGACCCGACATATAACACGTTTGCTGCAGCGCTGAACT 60
   I  H  S  A  D  P  K  D  P  T  Y  N  T  F  A  A  A  L  N
TTATCGGCAGCGACAATGCGTTAAAACTGATTGTTGCAGTTCTTGCCGGATTCATTGCAA 120
F  I  G  S  D  N  A  L  K  L  I  V  A  V  L  A  G  F  I  A
TGAGTATTGCGGACCGTCCGGGATTTGCTCCGGGTATGGTCGGCGGATTTATGGCTACGC 180
M  S  I  A  D  R  P  G  F  A  P  G  M  V  G  G  F  M  A  T
AGGCGAATGCCGGATTCTTAGGCGGGCTGATTGCCGGATTCTTAGCCGGTTATGTCGTGA 240
Q  A  N  A  G  F  L  G  G  L  I  A  G  F  L  A  G  Y  V  V
TCCTGCTGAAAAAGCTGTTCGTCTTTATCCCTCAGTCTCTTGACGGATTAAAACCTGTAT 300
I  L  L  K  K  L  F  V  F  I  P  Q  S  L  D  G  L  K  P  V
TGATTTACCCGCTACTCGGTATTTTTATCACAGGTGTCCTGATGCAGTTCGTCATTAACA 360
L  I  Y  P  L  L  G  I  F  I  T  G  V  L  M  Q  F  V  I  N
CACCTGTAGCAGCGTTTATGAATTTCTTAACAAATTGGCTTGAAAGCCTTGGAACAGGCA 420
T  P  V  A  A  F  M  N  F  L  T  N  W  L  E  S  L  G  T  G
ACCTTGTTTTAATGGGTATTATTTTAGGCGGTATGATGGCTATCGACATGGGCGGCCCGC 480
N  L  V  L  M  G  I  I  L  G  G  M  M  A  I  D  M  G  G  P
TCAATAAAGCGGCGTTCACTTTCGGAATTGCGATGATCGATGCCGGAAACTATGCGCCGC 540
L  N  K  A  A  F  T  F  G  I  A  M  I  D  A  G  N  Y  A  P
ACGCTGCCATCATGGCAGGCGGAATGGTTCCGCCGCTTGGAATCGCACTGGCGACAACAT 600
H  A  A  I  M  A  G  G  M  V  P  P  L  G  I  A  L  A  T  T
TCTTTAGACACAAATTCTCTAAACGTGACCGTGAAGCCGGTATTACGTGCTACTTCATGG 660
F  F  R  H  K  F  S  K  R  D  R  E  A  G  I  T  C  Y  F  M
GAGCCGCTTTCGTTACAGAAGGCGCTATCCCGTTTGCAGCGGCTGATCTTCGCGTCATCC 720
G  A  A  F  V  T  E  G  A  I  P  F  A  A  A  D  L  R  V  I
CTGCAGCCGTTATCGGTTCTGCGGTAGCGGGCGGACTGACAGAATTTTTCCGGGTAACGC 780
P  A  A  V  I  G  S  A  V  A  G  G  L  T  E  F  F  R  V  T
TTCCTGCACCGCACGGAGGAGTATTCGTTGCATTCATTACGAATCATCCGCTGCTCTACT 840
L  P  A  P  H  G  G  V  F  V  A  F  I  T  N  H  P  L  L  Y
TATTGAGCATCGTGATCGGAGCGATCGTGACGGCGGTTATCTTGGGAATCATCAAAAAGC 900
L  L  S  I  V  I  G  A  I  V  T  A  V  I  L  G  I  I  K  K
CGGTTGAAGAGAAATAAGCAAAAAAGCGCCCGTAAAGGGCGCTTTTTTCAATGATATACG 960
P  V  E  E  K  -
-35                -10
CATAGAAGCGTTATGTATTTTATAATGAGTTGTGATAAAGTAAAGATGGTACATGAACTG 1020
AAACAGTGATCGTCTAGGAGGAACCACGTTGACTGAAGAACAAAAACCTACTTCTGAAAA 1080
                          M  T  E  E  Q  K  P  T  S  E  K
ATCGGTAAAAAGAAAATCCAACACGTATTGGGAATGGGGAAAGGCCATCATTATAGCAGT 1140
  S  V  K  R  K  S  N  T  Y  W  E  W  G  K  A  I  I  I  A  V
CGCACTTGCGCTGTTAATCCGCCATTTCCTGTTTGAACCGTACTTAGTGGAAGGATCATC 1200
  A  L  A  L  L  I  R  H  F  L  F  E  P  Y  L  V  E  G  S  S
AATGTACCCGACATTACATGACGGTGAACGGCTGTTTGTCAACAAAAGCGTCAATTATAT 1260
  M  Y  P  T  L  H  D  G  E  R  L  F  V  N  K  S  V  N  Y  I
CGGCGAAATCGAGCGGGGGGACATCGTCATTATTAACGGCGATACATCGAAAGTCCATTA 1320
  G  E  I  E  R  G  D  I  V  I  I  N  G  D  T  S  K  V  H  Y
TGTAAAGCGGCTCATCGGAAAACCGGGAGAAACGGTTGAAATGAAAAATGACACGCTTTA 1380
  V  K  R  L  I  G  K  P  G  E  T  V  E  M  K  N  D  T  L  Y
TATAAACGGCAAAAAAATCGCAGAACCGTACCTCGCGAGCAATAAAAAAGAAGCGAAAAA 1440
  I  N  G  K  K  I  A  E  P  Y  L  A  S  N  K  K  E  A  K  K
ACTGGGAGTCAACCTGACGGGAGATTTTGGCCCCGTAAAGGTTCCGAAAGGCAAATACTT 1500
  L  G  V  N  L  T  G  D  F  G  P  V  K  V  P  K  G  K  Y  F
CGTCATGGGCGACAATCGGCTGAATTCAATGGACAGCCGGAACGGACTCGGTTTAATTGC 1560
  V  M  G  D  N  R  L  N  S  M  D  S  R  N  G  L  G  L  I  A
CGAAAACCGGATCGTCGGCACCTCGAAGTTTGTGTTCTTCCCGTTTCATGATATGCGTCA 1620
  E  N  R  I  V  G  T  S  K  F  V  F  F  P  F  H  D  M  R  Q
GACAAAATAAAAACGCCGCGCTGCCCGATTCGGACGGCCGGCGTTTTTTATTTGGCAAAC 1680
  T  K  -
GAGGCGGCGATGGCGATGAACAATACCGTTGCCAGCACTGATCCGACAATAGCCAGAACG 1720
CTGATAAAAAGATTCGCCTGCTGCTTCTCTTTGCCGCGTTTTTCAAGCATCCGTTTTGAA 1780
AAAAGATGCGAGAACGTATAAATGAGGGCGATTCCGACGTACAAGCCAATGTCACGTGCC 1840
GTAAAGCCTGTGACGGCCAGAAAAACCCCGGTAAAGAAAATCATAAGACAGTATTCTGTG 1900
AGTGTTAAAAGCTT                                                1914
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Fig. 2. The nt sequence of a 1914 bp DNA fragment of *B. amyloliquefaciens* and the derived aa sequences. The complete ORF2 encodes the putative SipS2(BA) protein. Sequencing was performed with the Sanger dideoxy method using the AutoRead Sequencing Kit on double stranded DNA. The sequencing reactions were analysed on an automated laser fluorescent sequencer (A.L.F., Pharmacia Biotech Europe GmbH). The nt sequences of the PCR amplified overlapping 5′- and 3′-fragments (as shown in Fig. 1) have been confirmed on at least three different cloned fragments. The deduced ORF starts from translation initiation codon TTG (nt 1049) and ends with stop codon TAA at nt 1628. A putative RBS and proposed promoter boxes are underlined. A 13 bp-dyad forming a stem-loop structure is marked by arrows. The ORF would encode a 193 aa-protein of about 21 kDa. The nt sequence has the EMBL GenBank/DDBJ databases accession No. Z33640.

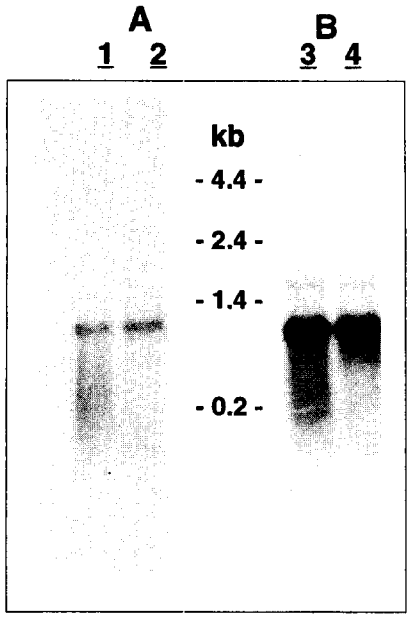

Fig. 3. Northern blot analysis of *sipS2(BA)* mRNA from *B. amyloliquefaciens*. Total RNA was isolated from exponential phase and early stationary phase cultures grown in Schaeffer sporulation medium [15,16] at 37°C. Approx. 10 μg of total RNA was electrophoresed on 1% agarose/formaldehyde gel and transferred onto supported nitrocellulose filters (Hybond C-super, Du Pont) and fixed by baking at 80°C. The probe labelling and the hybridisation were performed using an nick translation kit and rapid-hyb buffer (Amersham Life Science) according the instructions of the manufacturer. Ethidium bromide staining of the gel was performed to verify that equal amounts of RNA were present in each lane. *Legend:* Lanes A and B were probed with *sipS2(BA)* or *sipS1(BA)* core DNA, respectively. The lane 1 and 3 contain RNA from exponential phase, and lane 2 and 4 RNA from late exponential phase grown cultures.

the *B. subtilis* PTS [12,13]. The FruA proteins are membrane-bound proteins. Whether the *sipS2*(BA) gene is closely linked to the fruA gene in *B. amyloliquefaciens* and other *Bacilli* remains to be determined.

Northern blotting and E. coli sipS2(BA) gene expression. Northern blots were performed using RNA isolated from either exponential or late exponential phase *B. amyloliquefaciens* cultures. The core DNAs for either *sipS1(BA)* or *sipS2(BA)* were used as probes (Fig. 3). The

Table 2
The degree of homology between the aa sequences of different *Bacillus* SipS proteins [a]

Protein:	SipS1(BS)	SipS1(BL)	SipS1(BA)	SipS2(BA)
SipS1(BS)	–	69	83	68
SipS1(BL)	78	–	69	73
SipS1(BA)	90	79	–	66
SipS2(BA)	77	80	76	–

[a] The upper right-hand triangle gives the percentage of identical aa in a pairwise alignment, and the lower left hand triangle gives the corresponding values for identical plus chemically related aa residues. The comparisons were performed as described in the legend of Fig. 5. The abbreviations BS; BL, BA1 and BA2 are used for the SipS proteins of *B. subtilis*, *B. licheniformis* and of *B. amyloliquefaciens* as for Fig. 5.

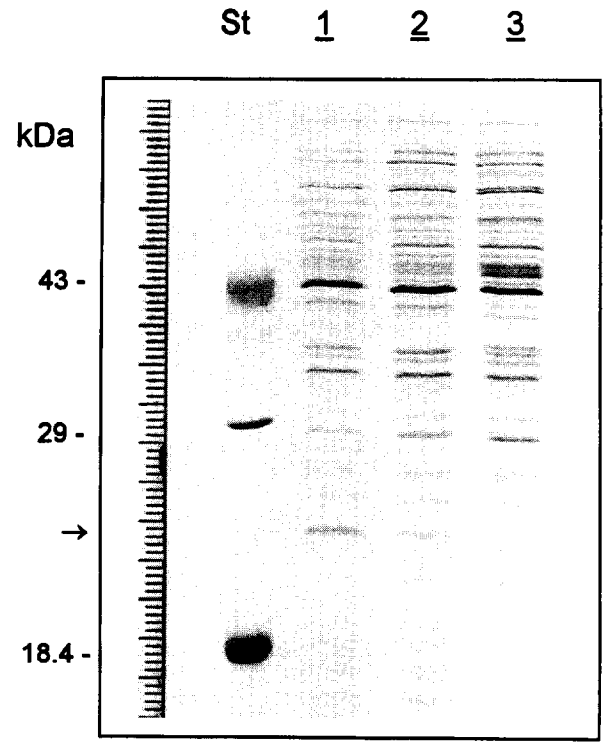

Fig. 4. Expression of the putative *sipS2(BA)* gene under the control *E. coli* using the P*spac* promoter. A DNA fragment encoding the promoterless gene sequence from nt 1005 to nt 1812 (Fig. 2) was PCR amplified from chromosomal DNA of *B. amyloliquefaciens* and cloned into vector pSPAC [14]. After transformation in *E. coli* DH5a [12], cells were grown in complex medium TBY [17] and either induced by IPTG (50 μM) in the exponential phase or not induced and grown for another 3 h at 37°C. The cells were harvested, sonicated and the soluble fractions (15 μl) applied to 10% SDS/PAGE gels [18]. The lanes 1 and 2 contain the proteins of an induced and non-induced culture, respectively. In lane 3 a sonicated cell extract of a control culture (without *sipS2(BA)* insert) was loaded. The standard proteins used were from Gibco BRL (Life Technologies Inc.). The arrow indicates the position of the putative SipS2 protein.

hybridisation signals indicated that both genes were indeed functional and transcribed at quite different expression levels from monocistronic transcription units (Fig. 3). To investigate this further we fused the minimal coding region of the putative *sipS2(BA)* gene (without putative promoter elements) downstream from the P*spac* promoter in the pSPAC vector [14] and transformed this construct into *E. coli*. After induction with IPTG, an additional protein band of about 21 kDa appeared in SDS-PAGE gels of sonicated cell extracts (Fig. 4). We ascribe the presence of this protein in SDS-PAGE gels of extracts from non-induced cultures to leakyness of the P*spac* promoter in *E. coli* [14]. The molecular weight of the inducible protein was close to that expected for the putative SipS2(BA) protein deduced from nt sequencing (Fig. 5). We therefore propose the existence of at least two type I SPase isozymes in *Bacillus amyloliquefaciens*, that are encoded by genes that did not cross-hybridise after Southern blotting (data not shown). Whether the two proteins have specialised func-

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SipS1(BS)    MKSEN--VSKK------KSILEWAKAIVIAVVLALLIRNFIFAPYVVDGD
SipS1(BA)    MKSEKEKTSKK------SAVLDWAKPIIIAVVLAVLIRNFLFAPYVVDGE
SipS1(BL)    MTEEKS-TNKK------NSLFEWVKAIIIAVVLALLIRAFLFEPYLVEGT
SipS2(BA)    MTEEQKPTSEKSVKRKSNTYWEWGKAIIIAVALALLIRHFLFEPYLVEGS
SipS1(BPu)   ----------------------------------------PYVVEGT
             *..*   ...*          .* *.*.***.**.*** *.*.**.*.*.

SipS1(BS)    SMYPTLHNRERVFVNMTVKYIGEFDRGDIVVLNGD--DVHYVKRIIGLPG
SipS1(BA)    SMEPTLHDRERIFVNMTVKYISDFKRGQIVVLNGE--NEHYVKRIIGLPG
SipS1(BL)    SMDPTLHDGERLFVYKTVRYVGEFKRGDIVIIDGDEKNVHYVKRLIGLPG
SipS2(BA)    SMYPTLHDGERLFVNKSVNYIGEIERGDIVIINGDTSKVHYVKRLIGKPG
SipS1(BPu)   SMDPTLHNTERVFVNKTVDYFGDYKRGQIIVLDGEDRSTHYVKRLIGLPG
             ** ****. **.** ..* * .. .**.*....*.  . *****.** **

SipS1(BS)    DTVEMKNDQLYINGKKVDEPYLAANKKRAKQDGFDHLTDDFGPVKVPDNK
SipS1(BA)    DTVQMKNDQLYINGKKVSEPYLAANKKKAKQDGYT-LTDDFGPVKVPDDK
SipS1(BL)    DTVQMKDDTLYINGKKVSEPYLSENRKEAEAVGVK-LTGDFGPVKVPEGK
SipS2(BA)    ETVEMKNDTLYINGKKIAEPYLASNKKEAKKLGVN-LTGDFGPVKVPKGK
SipS1(BPu)   DKIEMKNDQLYVNGKKVAEPYLAFNKKKAAADG-TLLTPDFGPLTVPKGK
             ....**.* **.****..****. *.* *   * . ** ****..**..*

SipS1(BS)    YFVMGDNRRNSMDSRNGLGLFTKKQIAGTSKFVFYPFNEMRKTN 184aa
SipS1(BA)    YFVMGDNRRNSMDSRNGLGLFTKKQIAGTSKFVFFPFNEIRKTK 185aa
SipS1(BL)    YFVMGDNRQRSMDSRNGLGLIDKKRVAGTSQFVFFPFNEIRKTD 186aa
SipS2(BA)    YFVMGDNRLNSMDSRNGLGLIAENRIVGTSKFVFFPFHDMRQTK 193aa
SipS1(BPu)   YFVMGD---------------------------------------- 112aa
             ******** .********** .....***.***.**...* *.
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Fig. 5. Alignment of the deduced aa sequence with the sequences of other of the SipS-like proteins identified in *B. subtilis* 168 [8], *B. amyloliquefaciens* ATCC 23844(BE71), *B. licheniformis* ATCC9789 (Hoang, V. and Hofemeister, J., unpublished data), or deduced from the nt sequences of a *sipS*-core DNA of *B. pumilus* strain B68 (Hoang, V. and Hofemeister, J., unpublished data). In this alignment the 'core' sequences are represented by the extension of the *B. pumilus* SipS1(BPu) sequence. Asterisks (*) represent identical and dashes (–) similar aa residues. The aa's printed in bold letters are strictly conserved in known SPases [4,6]. The alignment was done with the CLUSTAL program from the PC Gene program package. The EMBL GenBank/ DDBJ databases accession numbers for the nt relevant sequences used in the comparisons are: Z11847-SipS1(BS), Z27458-SipS1(BA), Z33640-SipS2(BA), and C75604-SipS1(BL).

tions during the processing of secreted proteins is now under investigation.

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